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Transgenic Organism

Field of the Invention

The present invention relates to a method for producing a transgenic cell and a transgenic organism.

Background to the Invention

The ability to introduce genes and/or other DNA sequences into the germline or somatic cells of organisms such as mammals is one of the greatest technical advances in recent biology. Such animals are said to be transgenic. When germline changes are involved, the results of genetic manipulation are inherited by the offspring of the animals and all cells of these offspring inherit the introduced gene and in some cases deleted DNA as part of their genetic make-up. Transgenic mammals have provided a means of studying gene regulation during embryogenesis and in differentiation, for studying the action of oncogenes, and for studying the intricate interactions of cells in the immune system. The whole animal is the ultimate assay system for manipulating genes which direct complex biological processes. In addition, transgenic animals provide exciting possibilities for expressing useful recombinant proteins and for generating precise animal models of human genetic disorders.

The production of transgenic animals is commonly done in one of two ways: by targeted insertion of DNA by homologous recombination in embryonic stem (ES) cells which is a labour intensive and time-consuming process, or by pronuclear injection of a fertilised ovum in which integration of DNA is random and may lead to an insertion of large tandem arrays of DNA which are unstable and subject to rearrangements and deletions in subsequent cell divisions. WO99/51755 discusses use of a retroviral expression vector comprising a nucleic acid encoding at least one ribozyme for production of a transgenic animal. No specific disclosure is made of the retrovirus used in the specific example. Mention is also made of the possibility of using an adenovirus, an adeno-associated virus, a lentivirus, a herpes simplex virus or a vaccinia virus. However there are no specific examples of the use of these viruses.

Thus, in recent years, retroviruses have been proposed for use in gene therapy. Essentially, retroviruses are RNA viruses with a life cycle different to that of lytic viruses. In this regard, when a retrovirus infects a cell, its genome is converted to a DNA form. In other words, a retrovirus is an infectious entity that replicates through a DNA intermediate. More details on retroviral infection etc. are presented later on.

There are many retroviruses and examples include: murine leukaemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV may be found from the NCBI Genbank (i.e. Genome Accession No. AF033819).

As indicated above, there has been considerable interest in the development of retroviral vector systems based on lentiviruses, a small subgroup of the retroviruses. This interest arises firstly from the notion of using HIV-based vectors to target anti-HIV therapeutic genes to HIV susceptible cells and secondly from the prediction that, because lentiviruses are able to infect non-dividing cells (Lewis & Emerman 1993 J.Virol. 68, 510), vector systems based on these viruses would be able to transduce non-dividing cells (e.g. Vile & Russel 1995 Brit. Med. Bull. 51, 12). Vector systems based on HIV have been produced (Buchschacher & Panganiban 1992 J.Virol. 66, 2731) and they have been used to transduce CD4+ cells and, as anticipated, non-dividing cells (Naldini *et al*, 1996 Science 272, 263). In addition lentiviral vectors enable very stable long-term expression of the

gene of interest. This has been shown to be at least three months for transduced rat neuronal cells. The MLV based vectors were only able to express the gene of interest for six weeks.

HIV-based vectors produced to date result in an integrated provirus in the transduced cell that has HIV LTRs at its ends. This limits the use of these vectors as the LTRs have to be used as expression signals for any inserted gene unless an internal promoter is used. The use of internal promoters has significant disadvantages. The unpredictable outcome of placing additional promoters within the retroviral LTR transcription unit is well documented (Bowtell *et al*, 1988 J.Virol. 62, 2464; Correll *et al*, 1994 Blood 84, 1812; Emerman and Temin 1984 Cell 39, 459; Ghattas *et al*, 1991 Mol.Cell.Biol. 11, 5848; Hantzopoulos *et al*, 1989 PNAS 86, 3519; Hatzoglou *et al*, 1991 J.Biol.Chem 266, 8416; Hatzoglou *et al*, 1988 J.Biol.Chem 263, 17798; Li *et al*, 1992 Hum.Gen.Ther. 3, 381; McLachlin *et al*, 1993 Virol. 195, 1; Overell *et al*, 1988 Mol.Cell Biol. 8, 1803; Scharfman *et al*, 1991 PNAS 88, 4626; Vile *et al*, 1994 Gene Ther 1, 307; Xu *et al*, 1989 Virol. 171, 331; Yee *et al*, 1987 PNAS 84, 5197). The factors involved appear to include the relative position and orientation of the two promoters, the nature of the promoters and the expressed genes and any selection procedures that may be adopted. The presence of internal promoters can affect both the transduction titers attainable from a packaging cell line and the stability of the integrated vector.

HIV and other lentiviral LTRs have virus-specific requirements for gene expression. For example, the HIV LTR is not active in the absence of the viral Tat protein (Cullen 1995 AIDS 9, S19). It is desirable, therefore, to modify the LTRs in such a way as to change the requirements for gene expression. In particular tissue specific gene expression signals may be required for some gene therapy applications.

HIV vectors have a number of significant disadvantages which may limit their therapeutic application to certain diseases. HIV-1 has the disadvantage of being a human pathogen carrying potentially oncogenic proteins and sequences. There is the risk that introduction of vector particles produced in packaging cells which express HIV gag-pol will introduce these proteins into the patient leading to seroconversion.

For these reasons, there is a need to develop lentiviral-based vectors which do not introduce HIV proteins into patients. The present invention overcomes this problem.

Thus, according to one aspect of the present invention there is provided a method of producing a transgenic cell comprising introducing into a cell a non-primate lentiviral expression vector comprising a nucleotide of interest (NOI).

The present invention provides an efficient way of producing transgenic animals and which overcomes any potential difficulties associated with the use of primate lentiviruses.

Preferably, the non-primate lentiviral expression vector is derived from EIAV, FIV, BIV, CAEV or MVV, with EIAV being particularly preferred.

One of the advantages of the present invention is that the expression vector can be introduced in vivo or ex vivo. In one embodiment the method is carried out in vitro. In another embodiment, the cell is in utero.

Several methods for introducing foreign DNA into the germline of mammals have been developed. The techniques allow the mixing of cells from different embryos, i.e. chimaera production, introducing pluripotent cells such as ES cells into developing embryos, micro-injecting DNA, and infection by retroviruses. Many of these techniques have the fundamental requirement of removing fertilised eggs or early embryos, culturing them in vitro and then returning them to foster mothers where further embryogenesis can proceed. In particular the production of transgenic animals by targeted insertion of DNA by homologous recombination in ES cells is a labour intensive and time-consuming process with, e.g. a turnaround time of 8 to 9 weeks from nuclear injection.

One major advantage of this embodiment of the present invention is the ability to avoid the need to remove, culture in vitro and then reimplementation. It also avoids the intensive and time-consuming production of recombinant ES cells.

Indeed, a vast number of genes of unknown function are now available following large scale gene sequencing programmes. To develop therapeutic products from novel genomic targets, it will be necessary to correlate biology with gene sequence information. The present invention provides an efficient and effective in vivo method for assisting in the validation of targets.

Another advantage of the present invention is its flexibility; the lentiviral vector can be introduced throughout the development of the organism. Thus in one embodiment the cell is a prenatal cell, which could an embryonic cell. In a particular aspect of this embodiment the embryonic cell is in utero. However, the method may be applied to any cell such as any somatic cell and also any cell which is capable of giving rise to a germ line change. Such cells include the germ cells, of course, but the present invention can also be applied to a cell which is involved either directly or indirectly in gametogenesis or fertilisation. We also include equivalent cells which are arrived at without direct fertilisation, e.g. through cell nuclear replacement techniques.

Preferably the cell is an oocyte, an oviduct cell, an ovarian cell, an ovum, an ES cell, a blastocyte, a spermatocyte, a spermatid, a spermatozoa, or a spermatogonia.

The method is not limited to a particular cell type, but the cell is preferably a eukaryotic cell, such as an animal, preferably mammalian, or yeast cell. Examples of cells to which the present invention is applicable include murine, human, porcine, bovine, simian, ovine, equine, avian, insect or reptile or piscine cell. The cell may be from, e.g., C. elegans or drosophila.

In one embodiment, the cell is from a non-human organism.

Preferably the lentiviral expression vector is pseudotyped.

Preferably the lentiviral expression vector does not contain any functional accessory genes.

The NOI may be operably linked to a constitutive, tissue-specific or an inducible promoter.

Preferably, the NOI encodes a therapeutic protein, is an antisense oligonucleotide, or encodes a ribozyme.

The lentiviral expression vector may be introduced into a target cell through administration via any convenient route of access, such as a cell of the umbilical cord, placenta, or amniotic fluid; or directly into an organ such as the uterus, gonad, brain, kidney, liver, heart, bone marrow, blood, central nervous system, or lung.

In accordance with another aspect of the present invention there is provided a transgenic organism which is generated from or obtainable by generation from a transgenic cell according to the present invention.

One problem associated with the production of transgenic animals for establishing disease models arises where the loss of expression in say a knock out mouse is lethal. In the methods of the present invention the NOI can be operably linked to a tissue-specific or an inducible promoter. This is particularly advantageous where ablation of gene expression is desired at a particular developmental stage or in a specific tissue.

The NOI may be expressed in the transgenic organism in a constitutive, tissue-specific or regulatable manner. Examples of cells where the NOI may be expressed include a cell of any organ or tissue, such as a cell of the brain, kidney, liver, heart, bone marrow, blood, central nervous system, or lung of said organism. The NOI may also be expressed at a particular developmental stage of the organism.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:

Description of the Figures

Figure 1 shows a section of mouse liver stained for the β-galactosidase marker gene 3 days after vector injection;

Figure 2 shows a section of mouse liver stained for the β-galactosidase marker gene 7 days after vector injection;

Figure 3 shows a section of mouse liver stained for the β-galactosidase marker gene 14 days after vector injection;

Figure 4 shows a transverse section of mouse liver stained for the β-galactosidase marker gene 14 days after vector injection;

Figure 5 shows a section of mouse liver stained for the β-galactosidase marker gene 28 days after vector injection;

Figure 6 shows a transverse section of mouse liver stained for the β-galactosidase marker gene 28 days after vector injection;

Figure 7 shows a section of mouse liver stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 8 shows a transverse section of mouse liver stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 9 shows a section of mouse heart stained for the β-galactosidase marker gene 7 days after vector injection;

Figure 10 shows a section of mouse heart stained for the β-galactosidase marker gene 14 days after vector injection;

Figure 11 shows a section of mouse heart stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 12 shows a transverse section of mouse heart stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 13 shows a section of mouse brain stained for the β-galactosidase marker gene 3 days after vector injection;

Figure 14 shows a section of mouse brain stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 15 shows a transverse section of mouse brain stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 16 shows a section of mouse lung stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 17 shows a transverse section of mouse lung stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 18 shows a section of mouse muscle stained for the β-galactosidase marker gene 14 days after vector injection;

Figure 19 shows a section of mouse muscle stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 20 shows a transverse section of mouse muscle stained for the β-galactosidase marker gene 79 days after vector injection;

Figure 21 shows a section of mouse kidney stained for the β-galactosidase marker gene 79 days after vector injection; and

Figure 22 shows a transverse section of mouse kidney stained for the β-galactosidase marker gene 79 days after vector injection.

Detailed Description of the Invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

The present invention relates to a method of producing a transgenic cell using a non-primate lentiviral expression vector and a transgenic organism which is obtainable from the transgenic cell or of which the transgenic cell forms part. More particularly, the present invention relates to a lentiviral vector useful in gene therapy and in the production of disease models. The development of disease models, e.g. transgenic "knockout" mice, has greatly benefited studies of gene function, with particular relevance in establishing mammalian models of genetic disease.

Gene therapy includes any one or more of: the addition, the replacement, the deletion, the supplementation, the manipulation etc. of one or more nucleotide sequences in, for example, one or more targeted sites - such as targeted cells. If the targeted sites are targeted cells, then the cells may be part of a tissue or an organ. General teachings on gene therapy may be found in Molecular Biology (Ed Robert Meyers, Pub VCH, such as pages 556-558).

By way of further example, gene therapy also provides a means by which any one or more of: a nucleotide sequence, such as a gene, can be applied to replace or supplement a defective gene; a pathogenic gene or gene product can be eliminated; a new gene can be added in order, for example, to create a more favourable phenotype; cells can be manipulated at the molecular level to treat cancer (Schmidt-Wolf and Schmidt-Wolf, 1994, Annals of Hematology 69:273-279) or other conditions - such as immune, cardiovascular, neurological, inflammatory or infectious disorders; antigens can be manipulated and/or introduced to elicit an immune response - such as genetic vaccination.

A transgenic organism is an organism which includes in at least one of its cells a nucleotide of interest (NOI). In one embodiment the cell is a germline cell. In another embodiment, the cell is a somatic cell. More particularly, the NOI has been introduced experimentally, e.g. using cDNA technology.

The NOI is commonly referred to as a "transgene", i.e. a gene that is inserted into the cell in such a way that ensures its function. When the gene is inserted into a germ line gene should function, replicate and be transmitted as a normal gene.

The present invention encompasses chimeras and mosaics.

A "chimera" is an organism composed of a mixture of genetically different cells.

A "mosaic" is an organism in which the transgene is incorporated into the genome after the first cell division. The organism will be mosaic as different cells will have different sites of integration.

A transgenic organism of the invention is preferably a multicellular eukaryotic organism, such as an animal or a plant, or a fungus, or a unicellular eukaryotic organism such as a yeast.

Then organism is preferably an animal, more preferably a mammal.

The present invention employs a non-primate lentiviral expression vector.

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a host cell for the purpose of replicating the vectors comprising a segment of DNA. Examples of vectors used in

recombinant DNA techniques include but are not limited to plasmids, chromosomes, artificial chromosomes or viruses.

The term "expression vector" means a construct capable of *in vivo* or *in vitro/ex vivo* expression.

The vector used in the present invention is capable of transducing a target non-dividing cell. One advantage of these feature is that since freshly isolated oocytes are quiescent transduction rates may be enhanced by the use of say lentiviral rather than retroviral vectors.

In a typical vector for use in the method of the present invention, at least part of one or more protein coding regions essential for replication may be removed from the virus. This makes the retroviral vector replication-defective. Portions of the retroviral genome may also be replaced by a library encoding candidate modulating moieties operably linked to a regulatory control region and a reporter moiety in the vector genome in order to generate a vector comprising candidate modulating moieties which is capable of transducing a target non-dividing host cell and/or integrating its genome into a host genome.

A "non-primate" vector, as used herein, refers to a vector derived from a virus which does not primarily infect primates, especially humans. Thus, non-primate virus vectors include vectors which infect non-primate mammals, such as dogs, sheep and horses, reptiles, birds and insects.

A lentiviral or lentivirus vector, as used herein, is a vector which comprises at least one component part derivable from a lentivirus. Preferably, that component part is involved in the biological mechanisms by which the vector infects cells, expresses genes or is replicated. The term "derivable" is used in its normal sense as meaning the sequence need not necessarily be obtained from a retrovirus but instead could be derived

therefrom. By way of example, the sequence may be prepared synthetically or by use of recombinant DNA techniques.

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al*1992 EMBO. J 11: 3053-3058; Lewis and Emerman 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

The non-primate lentivirus may be any member of the family of lentiviridae which does not naturally infect a primate and may include a feline immunodeficiency virus (FIV), a bovine immunodeficiency virus (BIV), a caprine arthritis encephalitis virus (CAEV), a Maedi visna virus (MVV) or an equine infectious anaemia virus (EIAV). Preferably the lentivirus is an EIAV. Equine infectious anaemia virus infects all equidae resulting in plasma viremia and thrombocytopenia (Clabough, et al. 1991. J Virol. 65:6242-51). Virus replication is thought to be controlled by the process of maturation of monocytes into macrophages.

EIAV has the simplest genomic structure of the lentiviruses and is particularly preferred for use in the present invention. In addition to the *gag*, *pol* and *env* genes EIAV encodes three other genes: *tat*, *rev*, and *S2*. *Tat* acts as a transcriptional activator of the viral LTR (Derse and Newbold1993 Virology. 194:530-6; Maury, et al 1994 Virology. 200:632-42) and Rev regulates and coordinates the expression of viral genes through rev-response elements (RRE) (Martarano et al 1994 J Virol. 68:3102-11). The mechanisms of action of these two proteins are thought to be broadly similar to the analogous mechanisms in the primate viruses (Martano et al ibid). The function of S2 is unknown. In addition, an EIAV protein, Ttm, has been identified that is encoded by the first exon of *tat* spliced to the *env* coding sequence at the start of the transmembrane protein.

In addition to protease, reverse transcriptase and integrase non-primate lentiviruses contain a fourth *pol* gene product which codes for a dUTPase. This may play a role in the ability of these lentiviruses to infect certain non-dividing cell types.

The viral RNA of the invention is transcribed from a promoter, which may be of viral or non-viral origin, but which is capable of directing expression in a eukaryotic cell such as a mammalian cell. Optionally an enhancer is added, either upstream of the promoter or downstream. The RNA transcript is terminated at a polyadenylation site which may be the one provided in the lentiviral 3' LTR or a different polyadenylation signal.

Thus the present invention employs a DNA transcription unit comprising a promoter and optionally an enhancer capable of directing expression of a non-primate lentiviral vector genome.

Transcription units as described herein comprise regions of nucleic acid containing sequences capable of being transcribed. Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition. The sequences may be in the sense or antisense orientation with respect to the promoter. Antisense constructs can be used to inhibit the expression of a gene in a cell according to well-known techniques. Nucleic acids may be, for example, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or analogues thereof. Sequences encoding mRNA will optionally include some or all of 5' and/or 3' transcribed but untranslated flanking sequences naturally, or otherwise, associated with the translated coding sequence. It may optionally further include the associated transcriptional control sequences normally associated with the transcribed sequences, for example transcriptional stop signals, polyadenylation sites and downstream enhancer elements. Nucleic acids may comprise cDNA or genomic DNA (which may contain introns).

The basic structure of a retrovirus genome is a 5' LTR and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and

gag, pol and *env* genes encoding the packaging components - these are polypeptides required for the assembly of viral particles. More complex retroviruses have additional features, such as *rev* and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell.

In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also serve as enhancer-promoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Preferred vectors for use in accordance with the present invention are recombinant non-primate lentiviral vectors.

The term "recombinant lentiviral vector" (RLV) refers to a vector with sufficient retroviral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome. The RLV carries non-viral coding sequences which are to be delivered by the vector to the target cell. An RLV is incapable of independent replication to produce infectious retroviral particles within the final target cell. Usually the RLV

lacks a functional *gag-pol* and/or *env* gene and/or other genes essential for replication. The vector of the present invention may be configured as a split-intron vector. A split intron vector is described in PCT patent application WO 99/15683.

Preferably the lentiviral vector of the present invention has a minimal viral genome.

As used herein, the term "minimal viral genome" means that the viral vector has been manipulated so as to remove the non-essential elements and to retain the essential elements in order to provide the required functionality to infect, transduce and deliver a nucleotide sequence of interest to a target host cell. Further details of this strategy can be found in our WO98/17815.

A minimal lentiviral genome for use in the present invention will therefore comprise (5') R - U5 - one or more first nucleotide sequences - U3-R (3'). However, the plasmid vector used to produce the lentiviral genome within a host cell/packaging cell will also include transcriptional regulatory control sequences operably linked to the lentiviral genome to direct transcription of the genome in a host cell/packaging cell. These regulatory sequences may be the natural sequences associated with the transcribed retroviral sequence, i.e. the 5' U3 region, or they may be a heterologous promoter such as another viral promoter, for example the CMV promoter. Some lentiviral genomes require additional sequences for efficient virus production. For example, in the case of HIV, *rev* and RRE sequence are preferably included. However the requirement for *rev* and RRE may be reduced or eliminated by codon optimisation. Further details of this strategy can be found in our WO01/79518.

In one embodiment of the present invention, the lentiviral vector is a self-inactivating vector.

By way of example, self-inactivating retroviral vectors have been constructed by deleting the transcriptional enhancers or the enhancers and promoter in the U3 region of the 3′ LTR. After a round of vector reverse transcription and integration, these changes are copied into both the 5′ and the 3′ LTRs producing a transcriptionally

inactive provirus (Yu *et al* 1986 Proc Natl Acad Sci 83: 3194-3198; Dougherty and Temin 1987 Proc Natl Acad Sci 84: 1197-1201; Hawley *et al* 1987 Proc Natl Acad Sci 84: 2406-2410; Yee *et al* 1987 Proc Natl Acad Sci 91: 9564-9568). However, any promoter(s) internal to the LTRs in such vectors will still be transcriptionally active. This strategy has been employed to eliminate effects of the enhancers and promoters in the viral LTRs on transcription from internally placed genes. Such effects include increased transcription (Jolly *et al* 1983 Nucleic Acids Res 11: 1855-1872) or suppression of transcription (Emerman and Temin 1984 Cell 39: 449-467). This strategy can also be used to eliminate downstream transcription from the 3′ LTR into genomic DNA (Herman and Coffin 1987 Science 236: 845-848). This is of particular concern in human gene therapy where it is of critical importance to prevent the adventitious activation of an endogenous oncogene.

In our WO99/32646 we give details of features which may advantageously be applied to the present invention. In particular, it will be appreciated that the non-primate lentivirus genome (1) preferably comprises a deleted *gag* gene wherein the deletion in gag removes one or more nucleotides downstream of about nucleotide 350 or 354 of the *gag* coding sequence; (2) preferably has one or more accessory genes absent from the non-primate lentivirus genome; (3) preferably lacks the *tat* gene but includes the leader sequence between the end of the 5' LTR and the ATG of *gag*; and (4) combinations of (1), (2) and (3). In a particularly preferred embodiment the lentiviral vector comprises all of features (1) and (2) and (3).

The non-primate lentiviral vector may be a targeted vector. The term "targeted vector" refers to a vector whose ability to infect/transfect/transduce a cell or to be expressed in a host and/or target cell is restricted to certain cell types within the host organism, usually cells having a common or similar phenotype.

Target cells for gene therapy using retroviral vectors include but are not limited to haematopoietic cells, (including monocytes, macrophages, lymphocytes, granulocytes, or progenitor cells of any of these); endothelial cells, tumour cells, stromal cells,

astrocytes, or glial cells, muscle cells, epithelial cells, neurons, fibroblasts, hepatocyte. astrocyte, kidney, liver, heart and lung cells.

The vector may be pseudotyped with any molecule of choice, including but not limited to envelope glycoproteins (wild type or engineered variants or chimeras) of VSV-G, rabies, Mokola, MuLV, LCMV, Sendai, Ebola.

As indicated above, a nucleotide sequence used in the method of the present invention is inserted into a vector which is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The NOI produced by a host recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used.

The heterologous gene, i.e. NOI, may be any allelic variant of a wild-type gene, or it may be a mutant gene. The term "gene" is intended to cover nucleic acid sequences which are capable of being at least transcribed. Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition. The sequences may be in the sense or antisense orientation with respect to the promoter. Antisense constructs can be used to inhibit the expression of a gene in a cell according to well-known techniques. Nucleic acids may be, for example, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or analogues thereof. Sequences encoding mRNA will optionally include some or all of 5' and/or 3' transcribed but untranslated flanking sequences naturally, or otherwise, associated with the translated coding sequence. It may optionally further include the associated transcriptional control sequences normally associated with the transcribed sequences, for example transcriptional stop signals, polyadenylation sites and downstream enhancer elements. Nucleic acids may comprise cDNA or genomic DNA (which may contain introns). However, it is generally preferred to use cDNA because it is expressed more efficiently since intron removal is not required.

Suitable NOI coding sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain

antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives therof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters.

Suitable NOIs for use in the present invention in the treatment or prophylaxis of cancer include NOIs encoding proteins which: destroy the target cell (for example a ribosomal toxin), act as: tumour suppressors (such as wild-type p53); activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); inhibitors of angiogenesis; or which provide enhanced drug sensitivity (such as pro-drug activation enzymes); indirectly stimulate destruction of target cell by natural effector cells (for example, strong antigen to stimulate the immune system or convert a precursor substance to a toxic substance which destroys the target cell (for example a prodrug activating enzyme)). Encoded proteins could also destroy bystander tumour cells (for example with secreted antitumour antibody-ribosomal toxin fusion protein), indirectly stimulate destruction of bystander tumour cells (for example cytokines to stimulate the immune system or procoagulant proteins causing local vascular occlusion) or convert a precursor substance to a toxic substance which destroys bystander tumour cells (e.g. an enzyme which activates a prodrug to a diffusible drug).

NOI(s) may be used which encode antisense transcripts or ribozymes which interfere with expression of cellular genes for tumour persistence (for example against aberrant *myc* transcripts in Burkitts lymphoma or against *bcr-abl* transcripts in chronic myeloid leukemia). The use of combinations of such NOIs is also envisaged.

For further information on the nature of therapeutic genes see WO95/21927 and WO98/15294.

Suitable NOIs for use in the treatment or prevention of ischaemic heart disease include NOIs encoding plasminogen activators. Suitable NOIs for the treatment or prevention of rheumatoid arthritis or cerebral malaria include genes encoding anti-inflammatory proteins, antibodies directed against tumour necrosis factor (TNF) alpha, and anti-adhesion molecules (such as antibody molecules or receptors specific for adhesion molecules).

Examples of hypoxia regulatable therapeutic NOIs can be found in WO95/21927.

The NOI coding sequence may encode a fusion protein or a segment of a coding sequence.

Instead of, or as well as, being selectively expressed in target tissues, the NOI or NOIs may encode a pro-drug activating enzyme or enzymes which have no significant effect or no deleterious effect until the individual is treated with one or more pro-drugs upon which the enzyme or enzymes act. In the presence of the active NOI, treatment of an individual with the appropriate pro-drug leads to enhanced reduction in tumour growth or survival.

A pro-drug activating enzyme may be delivered to a tumour site for the treatment of a cancer. In each case, a suitable pro-drug is used in the treatment of the patient in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the vector. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase); 5-fluorocytosine (with cytosine deaminase); doxorubicin-N-p-hydroxyphenoxyacetamide (with penicillin-V-amidase); para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); cephalosporin nitrogen mustard carbamates (with β-lactamase); SR4233 (with P450 Reductase); ganciclovir (with HSV thymidine kinase); mustard pro-drugs with nitroreductase and cyclophosphamide (with P450).

Examples of suitable pro-drug activating enzymes for use in the invention include a thymidine phosphorylase which activates the 5-fluoro-uracil pro-drugs capcetabine and furtulon; thymidine kinase from herpes simplex virus which activates ganciclovir; a cytochrome P450 which activates a pro-drug such as cyclophosphamide to a DNA damaging agent; and cytosine deaminase which activates 5-fluorocytosine. Preferably, an enzyme of human origin is used.

Suitable NOIs for use in the treatment or prevention of ischaemic heart disease include NOIs encoding plasminogen activators. Suitable NOIs for the treatment or prevention of rheumatoid arthritis or cerebral malaria include genes encoding anti-inflammatory proteins, antibodies directed against tumour necrosis factor (TNF) alpha, and anti-adhesion molecules (such as antibody molecules or receptors specific for adhesion molecules).

The expression products encoded by the NOIs may be proteins which are secreted from the cell. Alternatively the NOI expression products are not secreted and are active within the cell. In either event, it is preferred for the NOI expression product to demonstrate a bystander effect or a distant bystander effect; that is the production of the expression product in one cell leading to the killing of additional, related cells, either neighbouring or distant (e.g. metastatic), which possess a common phenotype.

Where macrophages or other haematopoietic cells are used, NOIs may be used which encode, for example, cytokines. These would serve to direct the subsequent differentiation of the haematopoietic stemp cells (HSCs) containing a therapeutic NOI. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF-β1, insulin, IFN-γ, IGF-I, IGF-II, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin α, Inhibin β, IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotactin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC

(69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β-NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1α, SDF1β, SCF, SCGF, stem cell factor (SCF), TARC, TGF-α, TGF-β, TGF-β2, TGF-β3, tumour necrosis factor (TNF), TNF-α, TNF-β, TNIL-1, TPO, VEGF, GCP-2, GRO/MGSA, GRO-β, GRO-γ and HCC1.

For some applications, a combination of some of these cytokines may be preferred, in particular a combination which includes IL-3, IL-6 and SCF, for the maintenance and expansion of stem cell populations. For differentiation *in vitro*, further cytokines may be added such as GM-CSF and M-CSF to induce differentiation of macrophages or GM-CSF and G-CSF to obtain neutrophils. On reintroduction of the engineered cells into the individual from whom they were derived, the body's own mechanisms then permit the cells or their differentiated progeny to migrate into the affected area e.g. the tumour.

Optionally, another NOI may be a suicide gene, expression of which in the presence of an exogenous substance results in the destruction of the transfected or transduced cell. An example of a suicide gene includes the herpes simplex virus thymidine kinase gene (HSV *tk*) which can kill infected and bystander cells following treatment with ganciclovir.

Optionally another NOI may be a targeting protein (such as an antibody to the stem cell factor receptor (WO-A-92/17505; WO-A-92/21766)). For example, recombinant (ecotropic) retroviruses displaying an antibody (or growth factor or peptide) against a receptor present on HSCs (CD34 or stem cell factor, for example) might be used for targeted cell delivery to these cells, either *ex vivo* by incubating unfractionated bone marrow with virus or by intravenous delivery of virus.

NOIs may also include marker genes (for example encoding β-galactosidase or green fluorescent protein) or genes whose products regulate the expression of other genes. In addition, NOIs may comprise sequences coding fusion protein partners in frame with a sequence encoding a protein of interest. Examples of fusion protein partners include the DNA binding or transcriptional activation domain of GAL4, a 6xHis tag and β-galactosidase. It may also be desirable to add targeting sequences to target proteins encoding by NOIs to particular cell compartments or to secretory pathways. Such targeting sequences have been extensively characterised in the art.

In one embodiment, at least one NOI, operably linked to a bacterial HRE according to the present invention encodes an oxygen-responsive bacterial transcriptional regulatory protein such as FNR. Such a construct will provide an autoregulated system since in the presence of hypoxia, expression of the bacterial transcriptional regulatory protein from the HRE construct will increase and serve to further increase transcription from the HRE construct and other HRE constructs present.

In one preferred embodiment, the NOI encodes a ribozyme. Ribozymes are RNA molecules that can function to catalyse specific chemical reactions within cells without the obligatory participation of proteins. For example, group I ribozymes take the form of introns which can mediate their own excision from self-splicing precursor RNA. Other ribozymes are derived from self-cleaving RNA structures which are essential for the replication of viral RNA molecules. Like protein enzymes, ribozymes can fold into secondary and tertiary structures that provide specific binding sites for substrates as well as cofactors, such as metal ions. Examples of such structures include hammerhead, hairpin or stem-loop, pseudoknot and hepatitis delta antigenomic ribozymes have been described.

Each individual ribozyme has a motif which recognises and binds to a recognition site in a target RNA. This motif takes the form of one or more "binding arms" but generally two binding arms. The binding arms in hammerhead ribozymes are the flanking sequences Helix I and Helix III which flank Helix II. These can be of variable length, usually between 6 to 10 nucleotides each, but can be shorter or longer.

The length of the flanking sequences can affect the rate of cleavage. For example, it has been found that reducing the total number of nucleotides in the flanking sequences from 20 to 12 can increase the turnover rate of the ribozyme cleaving a HIV sequence, by 10-fold (Goodchild, JVK, 1991 Arch Biochem Biophys 284: 386-391). A catalytic motif in the ribozyme Helix II in hammerhead ribozymes cleaves the target RNA at a site which is referred to as the cleavage site. Whether or not a ribozyme will cleave any given RNA is determined by the presence or absence of a recognition site for the ribozyme containing an appropriate cleavage site.

Each type of ribozyme recognizes its own cleavage site. The hammerhead ribozyme cleavage site has the nucleotide base triplet GUX directly upstream where G is guanine, U is uracil and X is any nucleotide base. Hairpin ribozymes have a cleavage site of BCUGNYR, where B is any nucleotide base other than adenine, N is any nucleotide, Y is cytosine or thymine and R is guanine or adenine. Cleavage by hairpin ribozymes takes places between the G and the N in the cleavage site.

More details on ribozymes may be found in "Molecular Biology and Biotechnology" (Ed. RA Meyers 1995 VCH Publishers Inc p831-8320 and in "Retroviruses" (Ed. JM Coffin et al 1997 Cold Spring Harbour Laboratory Press pp 683).

Expression of the ribozyme may be induced in all cells, but will only exert an effect in those in which the target gene transcript is present.

Alternatively, instead of preventing the association of the components directly, the substance may suppress the biologically available amount of a polypeptide of the invention. This may be by inhibiting expression of the component, for example at the level of transcription, transcript stability, translation or post-translational stability. An example of such a substance would be antisense RNA or double-stranded interfering RNA sequences which suppresses the amount of mRNA biosynthesis.

The NOI may be under the expression control of an expression regulatory element,

usually a promoter or a promoter and enhancer. The enhancer and/or promoter may be preferentially active in a hypoxic or ischaemic or low glucose environment, such that the NOI is preferentially expressed in the particular tissues of interest, such as in the environment of a tumour, arthritic joint or other sites of ischaemia. Thus any significant biological effect or deleterious effect of the NOI on the individual being treated may be reduced or eliminated. The enhancer element or other elements conferring regulated expression may be present in multiple copies. Likewise, or in addition, the enhancer and/or promoter may be preferentially active in one or more specific cell types - such as any one or more of macrophages, endothelial cells or combinations thereof. Further examples include include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated non-replicating cells such as macrophages and neurons.

The term “operably linked” means that the components described are in a relationship permitting them to function in their intended manner. A library comprising a regulatory sequence "operably linked" to a reporter sequence is ligated in such a way that expression of the nucleic acid reporter sequence is achieved under conditions compatible with the control sequences.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term “enhancer” includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

The promoter and enhancer of the transcription units encoding the secondary delivery system are preferably strongly active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary delivery system. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of temporally restricted

promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

In one preferred embodiment the combined use of a strong constitutive promoter such as CMV, or house-keeping promoter such as PGK, and the Tet-regulation system may be used for control of gene expression. In addition to the Tet system other inducible systems include the metallothionein, hsp68, lacZ, and SV40 T antigen systems.

Transactivating factors may be employed through use of two transgenic lines, namely one line which expresses the NOI under promoter "a", and a second line which expresses the transactivating factor "b" of promoter "a".

In another embodiment use may be made of the FLP recombinase system in which an inactive transgene is converted into the active form in a recombination event mediated by yeast FLP recombinase. Use may also be made of the bacteriophage P1 Cre recombinase system, which allows genes to be silenced in particular cell or tissue types and at specific times of the organisms development.

Ubiquitous expression may be achieved using promoters from housekeeping genes, such as beta-actin, mouse metallothionein, HMGCR and histone H4.

Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of an NOI in one tissue while remaining largely "silent" in other tissue types.

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

The level of expression of an NOI under the control of a particular promoter may be modulated by manipulating the promoter region. For example, different domains within a promoter region may possess different gene regulatory activities. The roles of these different regions are typically assessed using vector constructs having different variants of the promoter with specific regions deleted (that is, deletion analysis). This approach may be used to identify, for example, the smallest region capable of conferring tissue specificity.

A number of tissue specific promoters, described above, may be particularly advantageous in practising the present invention. In most instances, these promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

Promoters suitable for cardiac-specific expression include the promoter from the murine cardiac α-myosin heavy chain (MHC) gene. Suitable vascular endothelium-specific promoters include the Et-1 promoter and von Willebrand factor promoter.

Prostate specific promoters include the 5'flanking region of the human glandular kallikrein-1 (hKLK2) gene and the prostate specific antigen (hKLK3).

Examples of promoters/enhancers which are cell specific include a macrophage-specific promoter or enhancer, such as CSF-1 promoter-enhancer, or elements from a mannose receptor gene promoter-enhancer (Rouleux *et al* 1994 Exp Cell Res 214:113-119). Alternatively, promoter or enhancer elements which are preferentially active in neutrophils, or a lymphocyte-specific enhancer such as an IL-2 gene enhancer, may be used.

Moreover, the NOI may be placed under the control of one or more sequences which confer developmentally-regulated expression. This will result in the NOIs being activated at a given stage in the development of the transgenic organism or its progeny.

The development of transgenic 'knockout' mouse technology has greatly benefited studies of gene function, with particular relevance in establishing mammalian models of genetic disease. Current technology is, however, limiting in certain cases. For example many genes, often those of medical significance, are essential for viability. In such cases pups die during embryonic development or soon after birth. The present invention provides an effective transgenic method for regulatable gene ablation such that the production of a protein of interest may be switched off at the desired developmental stage, facilitating the generation of disease models in adult mammals. The transgenic organism can then be out through one or more of any phenotype screen. Suitable general and directed phenotypic screens include the use of fundus photography, blood pressure, behaviour analysis, X-ray fluoroscopy, dual-energy X-ray absorptiometry (DEXA), CAT scans, complete blood counts (CBC), urinalysis, blood chemistry, insulin levels, glucose tolerance, fluorescence-activated cell sorting (FACS), histopathology, expression data, developmental biology. The methodology of the present invention will have broad application in many areas where temporal gene regulation would be advantageous and in validating putative drug targets identified in genomics programmes.

The present invention may be used to modulate the expression of genes that are associated with human disease. A non-exhaustive list of genes is set out below (homologs of the genes are included):

Genes relating to cancer include, but are not limited to, *Cdh3, Ncam, Akp2, Asgr2, Bax, Bmp4, Ccnd1, Cd38, Cdc37, Cdkn1a, Cdkn1b, Cdkn1c, Csk, Epas1, Fgf2, Grpr, HBV, Igf1, Inhbb, Inpp5d, IRS1, Itga5, Kcna1, lacZ, Map2k4, Mdm2, Nfkbia, Ngfb, Oxt, Pemt, Plp, Shh, Src, Stat5a, Tcfap2a, Trp53, Blmh, Cd152, Cmkar2, Cmkbr5, Csf1, Csf3, Egfr, Gzmb, Ifng, Ifngr, IGFBP3, Il1r1, Il1rap, Il2, Il2ra, Il2rb, Il2rg, Il4, Il4ra, Il5, Il6, Il7r, Il10, Il12a, Il12b, Il12rb1, Il12rb2, IRS1, Kdr, Lifr, Lta, Ncam, Ntf3, Ntf5, Ntrk1, Ntrk2, Ntrk3, Ph, Prlr, Scya3, Smst, Tgfa, Tgfb1, Tgfb2, Tgfb3, Tnf, Tnfrsf1a, Tnfrsf1b, Tnfrsf5, Apc, Prkdc, TAg, Amh, Kit, Kitl, Ter, Fech, hr, Atm, E2f1, Hox11, Apc, Cdh3, Erbb2, Hras, Met, Notch4, PIP, PyVT, Tag, Wnt1, Madh3, Nf1,*

Ptch, Rb1, Odc, Bcl3, Fos, Fyn, Jun, Kras2, luc, Mos, Myc, Rab3a, Rela, Yes, Cd44, Mgmt, Plg, Ahr, Pgy2, Rag1, Btk, Igh-6, Jak3, Tcra, Tcrb, Tcrd, Ttp53, Ttpa, Vhlh and Wt1.

Genes relating to diabetes and obesity include, but are not limited to, *Ins2, Ins1, H2-Ea, H2-Ab1, Ifng, Prkdc, B2m, Rag1, Lep, Lepr, Cpe, Gck, Irs1, Irs2, Irs3, Irs4, Slc2a1, Cre, Dgat, tub, Pcsk2, Insr, Nos1, Nos3, Tnf, B2m, Thy1, Pomc, Ppara and Csf2.*

Genes relating to diseases of the cardiovascular system include, but are not limited to, *Acact, Alox15, Apoa2, Apob, Apoe, Ath1r, Cdkn1a, Cyp7a1, Epas1, Lcat, Ldlr, Pemt, Soat1, fld, hr, Ace, Adra1b, Adrb2, Adrbk1, Anx6, Atp7a, Cdh2, Evi1, Fn1, Gja1, Itga4, Jup, Kif3a, Nf1, Nos3, Nppa, Thra, Vcam1, Wt1, Agt, Bdkrb2, Bmp4, Drd3, Kcna1, Npr3, Ren, Apoc1, Apoc2, Apoc3, Apoa1, Cetp, Hpl, Lipc, Srb1, Adra2a, Agtr1a, Fgf2, Tnf, Asgr2, Lrpap1, Vldlr, Col3a1 and Plg.*

Genes relating to diseases of the endocrine system include, but are not limited to, *A, Cpe, fld, Insr, Lep, Lepr, tub, Acact, acd, Cacnb4, Crh, Foxn1, gl, Bmp4, Csf1, dwg, fsn, Hcph, Kit, Kitl, Mitf, oc, Phex, Prlr, Sparc, Grpr, Amh, Ar, Cga, Fshb, jsd, Ghrhr, Hmgic, Myo5a, Nr5a1, Oxt, p, Pit1, Prop1, Smst, Agt, Igf1, Gck, Pcsk2, Egfr, Foxn1, Mc1r, Tgfa, Thrb, Tshr and Ttr.*

Genes relating to apoptosis include, but are not limited to, *Fas, Ngfr, Tnfrsf1a, Tnfrsf1b, Bax, Bcl2, E2f1, Mdm2, Pcc, Rb1, Trp53, Bdnf, Fasl, Gzmb, Ntf3, Ntf5, Pfp, Tag and Tnf.*

Genes relating to immunology and inflamation include, but are not limited to, *Cd1, Cd3e, Cd3z, Cd4, Cd44, Cd5, Cd8a, Cd8b, Cd14, Cd152, Cd28, Cd38, Fcer1g, Fcgr2a, Fcgr2b, Fcgr3, Gpi1, H2-Aa, H2-DMa, H2-Eb1, H2-Eb2, H2, Hc, Icam1, Igh-1, Igh-5, Igh, Igk-C, Igl-1, Igl-5, Itga4, Itga5, Itgb2, Itgp, Lyst, Mar1, Ncam, PCC, Pep3, Ptprc, Ptprcap, PVR, Sele, Sell, Selp, Spn, Tapbh, Tcra, Tcrb, Tcrd, Thy1, Tlx1, Tnfrsf5, Tnfrsf6, Tnfsf5, Bmp4, Cmkar2, Cmkbr5, Csf1, Csf3, Egfr, Gzmb, Ifng, Ifngr, Il1r1, Il1rap, Il2, Il2ra, Il2rb, Il2rg, Il4, Il4ra, Il5, Il6, Il7r, Il10, Il12a, Il12b, Il12rb1, Il12rb2, Il15ra, Irs1, Itgb7, Kdr, Kitl, Lifr, Lta, Map2k4, Ntf3, Ntf5, Ntrk1, Ntrk3, Ph, Scya3, Smst, Tgfa, Tgfb1, Tgfb2, Tgfb3, Tnf, Tnfrsf1a, Tnfrsf1b, A, Atm, C3, C4,*

Cacnb4, Cd80, Cd86, Dh, Dsg3, Eef1a2, gl, hr, Lama2, Lbp, Lep, Lepr, Mitf, Pit1, Prop1, Scn8a, Abcb2, Ada, B2m, Bcl2, Bcl3, Btk, C2ta, Foxn1, H2-Ab1, Hcph, Igh-6, Igh-J, Ii, Jak3, Kit, Lck, Ltb, Lyn, Nfkb1, Nfkbia, Pfp, Pnlliprp2, Prkdc, Ptprcap, Rag1, Relb, Stat4, Stat6, Tlr4, Alox5, Alox5ap, Alox15, Bdkrb2, Blmh, Bmp6, Cmo, Crh, Nos2, Ptgs2, Vr1, Bax, E2f1, Inpp5d, Rb1, Stat5a, Trp53, Fyn and Irf1.

Genes relating to neurobiology include, but are not limited to, *Apoe, Atm, Bdnf, Cdk5, Chrna7, Cmkar4, Cstb, Gad2, Gfap, Gria2, Grik2, HD, Hdh, Nos1, Ntf3, Penk-rs, Prkcc, Psen1, Snca, Tnf and Vr1.*

In addition to the therapeutic gene or genes and the expression regulatory elements described, the delivery system may contain additional genetic elements for the efficient or regulated expression of the gene or genes, including promoters/enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. Expression levels may be improved by incorporating elements such as the WPRE.

The delivery of one or more one or more therapeutic genes by a delivery system according to the present invention may be used alone or in combination with other treatments or components of the treatment. In a further preferred embodiment of the first aspect of the invention, one or more nucleotides of interest (NOI) is introduced into the vector at the cloning site. Such therapeutic genes may be expressed from a promoter placed in the retroviral LTR or may be expressed from an internal promoter introduced at the cloning site.

For example, the delivery system of the present invention may be used to deliver one or more NOI(s) useful in the treatment of the disorders listed in WO98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-

thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

In addition, or in the alternative, the delivery system of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the delivery system of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune

response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders,

myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplanTation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

The subject treated by the method of the present invention may be an animal subject. Preferably the subject is a mammalian subject, more preferably a human subject.

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the delivery system of the present invention and optionally comprising one or more deliverable therapeutic and/or diagnostic NOI(s). Since the delivery system is a viral delivery system then the composition may in addition or in the alternative comprise a viral particle produced by or obtained from same. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular individual.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or

diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The delivery of one or more therapeutic genes by a delivery system according to the invention may be used alone or in combination with other treatments or components of the treatment.

The non-primate lentiviral vector particles of the present invention are typically generated in a suitable producer cell. Producer cells are generally mammalian cells but can be for example insect cells. A producer cell may be a packaging cell containing the virus structural genes, normally integrated into its genome. The packaging cell is then transfected with a nucleic acid encoding the vector genome, for the production of infective, replication defective vector particles. Alternatively the producer cell may be co-transfected with nucleic acid sequences encoding the vector genome and the structural components, and/or with the nucleic acid sequences present

on one or more expression vectors such as plasmids, adenovirus vectors, herpes viral vectors or any method known to deliver functional DNA into target cells.

The lentiviral vector may be used to deliver an NOI to any prenatal cell. The term "prenatal" means ocurring or present before birth. In one embodiment the method is applied to a cell at the embryonic stage. The term embryo includes animals in the early stages of development up to birth (or hatching). As used herein the term "embryo" includes "pre-embryo", i.e. the structure formed after fertilisation of an ovum but before differentiation of embryonic tissue, and includes a zygote and blastocyte. The term also includes a fetal cell, i.e. an embryonic cell which is in the latter stages of development. The present invention also encompasses delivery to a perinatal cell. The term "perinatal" refers to the period from about 3 months before to about one month after birth, and includes the neonatal period. The term "neonate" refers to the first few weeks following birth.

Generally the lentiviral vector may be used to deliver an NOI to any germ cell, including a primordial germ cell, or cell which is capable of giving rise to a germ line change. The term "germ cell" is the collective term for cells in the reproductive organis of multicellular organisms that divide by meiosis to produce gametes. The term "gametes" refers to the haploid reproductive cells - in effect the ovum and sperm. However, as indicated above the present invention is also applicable to cells involved in gametogenesis and cells from structures in which gametogenesis take place, such as the ovary.

Gametogenesis will now be described in relation to mammals by way of example only. The lentiviral vector may be used to deliver an NOI to any of the cells of structures mentioned below. It will be appreciated that the equivalent processes in non-mammalian organisms are also included in the present invention. In brief, gametogenesis is the process of forming gametes (by definition haploid, n) from diploid cells of the germ line. Spermatogenesis is the process of forming sperm cells by meiosis (in animals, by mitosis in plants) in specialized organs known as gonads (in males these are termed testes). After division the cells undergo differentiation to

become sperm cells. Oogenesis is the process of forming an ovum (egg) by meiosis (in animals, by mitosis in the gametophyte in plants) in specialized gonads known as ovaries.

In spermatogenesis the sperms are formed from the male germ cells, spermatogonia, which line the inner wall of the seminiferous tubules in the testis. A single spermatogonium divides by mitosis to form the primary spermatocyte, each of which undergoes the initial division of meiosis to form two secondary permatocytes. Each of these then undergoes a second meiotic diviion to form two spermatids, which mature into spermatozoa. The testis is composed of numerous seminiferous tubules, in whose walls spermatogenesis takes place. The primordial germ cells are formed in the germinal epithelium lining towards the outside of the tubule, and as cell divisions proceed the daughter cells move towards the lumen of the tubule. All these cells are nourished and supported by neighbouring Sertoli cells.

In oogenesis a primary oocyte is formed by differentiation of an oogonium and then undergoes the first division of meiosis to form a polar body and a secondary oocyte. Following fertilisation of the egg, the secondary oocyte undergoes the second meiotic division to form the mature ovum and a second polar body. The ovary contains many follicles composed of a developing egg surrounded by an outer layer of follicle cells. After ovulation the egg moves down the oviduct to the uterus.

It will be appreciated that the lentiviral vector may be administered at one locality, but the NOI is expressed or its effects felt, in another cell of the organism, i.e. the site of administration may be different from the target cell. Cells into which the non-primate lentiviral vector may be administered include the examples of target cells listed above. More preferably, the cell is at the embryonic stage, and for example is in utero, the lentiviral vector may be administered via the umbilical cord, placenta, or amniotic fluid, or by the intraperitoneal or intrahepatic routes. The introduction of the lentiviral vector is aided by the use of ultrasound.

The production of transgenic animals, using ES cells and otherwise, is well known in the art, and described for example in Manipulating the Mouse Embryo, 2nd Ed., by B. Hogan, R. Beddington, F. Costantini, and E. Lacy. Cold Spring Harbor Laboratory Press, 1994; Transgenic Animal Technology, edited by C. Pinkert. Academic Press, Inc., 1994; Gene Targeting: A Practical Approach, edited by A. L. Joyner. Oxford University Press, 1995; Strategies in Transgenic Animal Science, edited by G. M. Monastersky and J. M. Robl. ASM Press, 1995; and Mouse Genetics: Concepts and Applications, by Lee M. Silver, Oxford University Press, 1995. A useful general textbook on this subject is Houdebine, Transgenic animals – Generation and Use (Harwood Academic, 1997) – an extensive review of the techniques used to generate transgenic animals from fish to mice and cows.

Thus, for example the present invention permits the introduction of heterologous DNA into, for example, fertilised mammalian ova by lentiviral infection. In one embodiment the fertilised egg is collected from a donor mother at the one cell stage and the transduced cell is transferred to a foster mother. Integration which occurs at the one cell stage produces an organism which is a true transgenic, i.e. transgenic throughout, including the germ cells. If integration occurs at a later stage mosaics are produced. In a highly preferred method, developing embryos are infected with a lentivirus containing the desired DNA, and transgenic animals produced from the infected embryo. Traditional transgenic methods have required that the embryonic cells are transformed ex vivo then reimplanted into the uterus. A significant advantage associated with the present invention is that the NOI can be introduced in utero. Another method which may be used to produce a transgenic animal involves introducing a nucleic acid into pro-nuclear stage eggs by lentiviral infection. Injected eggs are then cultured before transfer into the oviducts of pseudopregnant recipients.

By way of a specific example for the construction of transgenic mammals, such as cows, nucleotide constructs comprising a sequence encoding a therapeutic protein are introduced using the method of the present invention into oocytes which are obtained from ovaries freshly removed from the mammal. The oocytes are aspirated from the

follicles and allowed to settle before fertilisation with thawed frozen sperm capacitated with heparin and prefractionated by Percoll gradient to isolate the motile fraction.

The fertilised oocytes are centrifuged, for example, for eight minutes at 15,000 g to visualise the pronuclei for injection and then cultured from the zygote to morula or blastocyst stage in oviduct tissue-conditioned medium. This medium is prepared by using luminal tissues scraped from oviducts and diluted in culture medium. The zygotes must be placed in the culture medium within two hours following microinjection.

Oestrous is then synchronized in the intended recipient mammals, such as cattle, by administering coprostanol. Oestrous is produced within two days and the embryos are transferred to the recipients 5-7 days after estrous. Successful transfer can be evaluated in the offspring by Southern blot.

Alternatively, the desired constructs can be introduced into embryonic stem cells (ES cells) and the cells cultured to ensure modification by the transgene. The modified cells are then injected into the blastula embryonic stage and the blastulas replaced into pseudopregnant hosts. The resulting offspring are chimeric with respect to the ES and host cells, and nonchimeric strains which exclusively comprise the ES progeny can be obtained using conventional cross-breeding. This technique is described, for example, in WO91/10741.

Analysis of animals which may contain transgenic sequences would typically be performed by either PCR or Southern blot analysis following standard methods. If desired, the organism can be bred to homozygosity.

The use of the present invention to produce transgenic organism for use in gene therapy and in the production of disease models has been mentioned above. In particular, disease models allow experimental investigation of gene function. In general transgenic organisms expressing novel genes or genes with a heterologous promoter represent gain-of-function mutations. Loss-of-function mutations can be created by gene targetting to create so-called "knockout" organisms. Transgenic organisms are also useful for the investigation of control regions and expression patterns. Transgenic organisms can also be used to identify novel genes using techniques such as insertional mutation, gene traps and promoter traps. Transgenic animals also have agricultural applications, for example to bring genetic improvements to milk yield, body mass, milk composition, disease resistance etc. Transgenic animals are also useful in so-called pharmaceutical farming in which transgenic livestock are used a bioreactors for the production of therapeutic proteins.

By way of example, the regulated ablation of SMN (homozygous deletion of which results in pre-natal mortality) would provide a useful model of spinal muscular atrophy for gene therapy studies. A CFTR deficiency model is also a valuable application. Other putative candidates include: presenilin-1, RARα, BDNF, VEGF and EGFR.

The analysis of resultant phenotypes can be carried out using standard techniques such as histological tissue analysis and microarray gene expression profiling.

The present invention will now be described by way of further example with reference to the following non-limiting Examples:

Example 1 - EIAV transduction of perinatal animals

An EIAV vector was injected into the umbilical vein of day 15 mice. Pups were born around 3 days post-injection at 18-19 days after conception. Mice were sacrificed at various stages of development (3, 7, 14 , 28 and 79 days) and samples prepared for

histology. Staining for the β-galactosidase marker gene expressed by the vector showed transduction of a number of organs including liver, lung, heart, muscle, kidney and brain. The results are shown in the Figures.

Expression levels did not decrease over the period of the study and clonal expansion of transduced cells was observed.

In addition to injection into the umbilical vein, injection directly into the circulation, CSF or other tissue may be carried out, or into the amniotic fluid. The latter may be particularly appropriate when transduction of lung or skin tissue is desired.

Example 2 – Haemophilia

This Example is carried out following the methodology of Example 1. Haemophilia is a blood condition in which an essential clotting factor is either partly or completely missing. It is an X-linked recessive disorder. There are two types of haemophilia, the most common being haemophilia A, in which Factor VIII is lacking. In haemophilia B, Factor IX is lacking. EIAV is used to deliver factor VIII or IX by EIAV to the umbilical vein of haemophiliac foetus or hepatic portal vein of perinates.

Example 3 - Cystic fibrosis

This Example is carried out following the methodology of Example 1. Cystic fibrosis is an hereditary recessive disorder caused by mutation of cystic fibrosis transmembrane conductance regulator (CFTR), a protein that is thought to have a role in ion transport, mucus rheology, inflammation and bacterial adherence. EIAV is used to deliver CFTR by to the amniotic fluid for transduction of lung.

Example 4 - Muscular dystrophy

This Example is carried out following the methodology of Example 1. Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder. DMD results from genetic deficiency in the level and/or activity of the protein dystrophin in the striated

musculature. EIAV is used to deliver of minidystrophin cDNA (corresponding to a mild Becker muscular dystrophy (BMD) phenotype) to the umbilical vein of perinates and/or directly into foetal skeletal muscle.

Example 5 – Ribozyme

This Example is carried out following the methodology of Example 1. A ribozyme which targets a gene on the biosynthetic pathway that generates melanin is delievered used EIAV. This approach facilitates the identification of transgenics.

Example 6 - Use of EIAV for transgenic models of Parkinson's.

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, affecting almost 2% of the population over 65. The disease is characterised by a movement disorder - parkinsonism - symptoms of which are rigidity, resting tremor and bradykinesia (slowness to initiate and carry out movement) . This results from the loss of neurons in the substantia nigra that produce the neurotransmitter dopamine. The causes of PD are largely unknown, although there are a few rare families in which the disease is inherited. In families with autosomal dominant PD two different missense mutations have been mapped in α-synuclein (Polymeropoulos et al 1997; Kruger et al 1998), which is a small phosphoprotein thought to be involved in synaptic vesicle transport. In the case of autosomal recessive juvenile parkinsonism (AR-JP), which develops in adolescence, Kitada et al (1998) showed the gene responsible to be Parkin, an E3 ubiquitin ligase recently proposed to catalyse the ubiquitination of α-synuclein (Shimura et al 2001). It has therefore been suggested that an inability to degrade α-synuclein results in AR-JP and possibly sporadic PD (Haass and Kahle 2001).

The EIAV vector system is used to deliver one or more of the following to mouse spermatogonial stem cells (Nagano et al 2001):

1. ribozyme to Parkin

2. mutant α-synuclein allele

3. ribozyme to tyrosine hydroxylase (enzyme required for dopamine synthesis)

Example 7 - Angiogenesis

The hypoxia inducible factor (HIF) is a transcriptional complex that plays a central role in oxygen homeostasis. The alpha subunit of HIF is targeted for degradation under normoxic conditions by the von Hippel-Lindau ubiquitylation complex that recognizes a hydroxylated proline residue in HIF. Steady state levels of the protein are consequently low and the transcriptional complex cannot form. A family of prolyl-4-hydroxylases have recently been described (Epstein at al 2001) whose enzyme activity is modulated by hypoxia, iron chelation and cobaltous ions, fulfilling the requirements for being oxygen sensors that regulate HIF. Suppression of proly-4-hydroxylase in cultured Drosophila melanogaster cells by RNA interference resulted in elevated expression of a hypoxia-inducible gene under normoxic conditions (Bruick and McKnight 2001).

The EIAV vector system is used to deliver:

1. A ribozyme to prolyl-4-hydroxlase (or VHL). This may lead to constitutive up-regulation of HIF-1alpha subunits, activation of the HIF complex and overexpression of HIF target genes.

2. Constitutively active HIF-1 (upregulation of HIF in normoxia) or PHD3 (downregulation of HIF in hypoxia).

to mouse oocytes by injection into the perivitelline space (Chan et al 1998; 2001).

The production and applications of transgenic mouse models in health-related research are well documented. The proposed research will enable the development of models

for a broad range of human diseases the generation of which are currently unmet by existing 'knockout' methodology.

Advantages over existing technology include the following:

1) Increased efficiency of transgene delivery by lentiviral transduction as compared with non-homologous recombination of injected DNA. Pronuclear injection leads to insertion of large tandem arrays of DNA which are unstable and subject to rearrangements and deletions. Lentiviral transduction generally leads to the stable integration of a limited number of vector copies distributed as discrete cassettes in the chromosomal DNA .

2) Reduction in turnaround time compared to current 'knock-out'. To produce mice with homozygous gene deletions is a relatively labour-intensive and time-consuming process requiring the cross-breeding of mosaic heterozygotes in which the engineered gene deletion has 'gone germline'. In contrast, by transducing oocytes prior to fertilisation, every cell will contain the ablation cassette. The need for cross-breeding is by-passed resulting in shorter turnaround times and a substantial decrease in the overall number of animals required.

3) Flexibility of gene product knock-down. As discussed this technology will be of particular value in establishing disease models where deletion of the gene of interest is lethal. It will be advantageous in all studies where ablation of gene expression is desired at particular developmental stages or restricted to specific tissues.

4) HIV vectors have a number of significant disadvantages which may limit their therapeutic application to certain diseases. HIV-1 has the disadvantage of being a human pathogen carrying potentially oncogenic proteins and sequences. There is the risk that introduction of vector particles produced in packaging cells which express HIV gag-pol will introduce these proteins into an individual leading to seroconversion. The present non-primate lentiviral-based vectors do not introduce HIV proteins into individuals.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in chemistry or biology or related fields are intended to be covered by the present invention. All publications mentioned in the above specification are herein incorporated by reference.

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